Approaches to the Evaluation of Chemical-induced Immunotoxicity

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The immune system plays a crucial role in maintaining health; however, accumulating evidence indicates that this system can be the target for immunotoxic effects caused by a variety of chemicals including the environmental pollutants of polychlorinated biphenyls, chlorinated dibenzo-*p*-dioxins, pesticides, and heavy metals. Adverse chemical-induced immunomodulation, which is studied within the discipline of immunotoxicology, may be expressed either as immunosuppression/immunodepression or immunoenhancement. The former may be manifested either as decreased resistance to opportunistic viral, bacterial, fungal, and other infectious agents or increased susceptibility to cancer. Immunoenhancement on the other hand may either increase the risk of autoimmune reactions or result in allergic reactions. This paper attempts to integrate several aspects of the immune system that are relevant to the assessment of potentially immunotoxic chemicals. — Environ Health Perspect 103(Suppl 9):17–22 (1995)

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Introduction

This review provides a general overview of immunotoxicology. The immune system evolved to protect the host from potentially pathogenic agents including microorganisms (viruses and bacteria), parasites, and fungi; to eliminate neoplastic cells; and to reject nonself components (*1*). Differentiated lymphoid organs first appear in the primitive forms of fish, with an increase in structural definition through amphibians and reptiles to birds and mammals. Consequently, several biomarkers of the immune response can be specific for all vertebrates (*2*). From the toxicologic point of view, the immune system can be a target for toxic effects of chemicals, therapeutic drugs, or any other foreign substances called xenobiotics (*3*). Chemically induced immunotoxic effects are investigated within the discipline of immunotoxicology (*4–8*). According to the recommendations of the International Seminar on the Immunological System as a Target for Toxic Damage:

> "a chemical substance should be considered immunotoxic when undesired events of the chemical are: (i) a direct and/or indirect action of the xenobiotic (and/or its biotransformation product) on the immune system; or (ii) an immunologically based host response to the compound and/or its metabolite(s), or host antigens are modified by the compound or its metabolite(s)" (*9*).

The adverse immunotoxic effects of xenobiotics include organ damage of the immune system, such as necrosis; multiple histopathologic effects in the thymus, the bone marrow, and the lymph nodes; chemical-induced cellular pathology, including abnormal proliferation of stem cells in the bone marrow; altered maturation of immunocompetent cells and changes in B- and T-cell subpopulations; functional alterations of immunocompetent cells generally classified as altered humoral-mediated immunity (HMI), cell-mediated immunity (CMI), or nonspecific responses (NSR); two-directional interaction of the immune system with xenobiotic detoxification and biotransformation mechanisms generally observed as an impairment of chemical elimination in immunodeficient individuals; xenobiotic-related *in vivo* functional immunopathology, such as weakened host resistance to viral and bacterial infections and to parasitic infestations; and altered immune surveillance mechanisms leading to increased incidence of cancer (*10,11*). Thus, structural and functional alterations of the immune system may lead to immunodepression/immunosuppression, which may modify the host defense mechanisms against infection and cancer, and induction of abnormal immune responses resulting in allergy and autoimmunity (*4–8,12–14*).

Diversity of Immunotoxic Effects

A number of chemical-induced immunotoxic effects have been reported; these effects can be organ specific, cell specific, immune function specific, they can be secondary following toxic effects of other organs, or they can be nonspecific (*4,5,13–17*). A brief classification of these immunotoxic effects is presented below in relation to the effects of currently recognized immunotoxic chemicals.

Alterations of the normal immune response usually result in increased susceptibility to viral, bacterial, or parasitic infections and to cancers. The type of adverse effect produced in the immune system upon exposure to a xenobiotic characterizes that xenobiotic as immunosuppressive or immunodepressive or as immunopotentiating.

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Abbreviations used: BRMs, biologic response modifiers; CMI, cell-mediated immunity; CPS, cyclophosphamide; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; HMI, humoral-mediated immunity; IgG, immunoglobulin G; IgE, immunoglobulin E; NK, natural killer cells; NSR, nonspecific responses; PBBs, polybrominated biphenyls; PCBs, polychlorinated biphenyls; PFC, plaque-forming cell; PLN, popliteal lymph node; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; OECD, Organization of Economic Cooperation for Development.

Immunosuppression and immunodepression are detected as marked decreases in immune function measured as an effect on humoral, cellular, or nonspecific parameters of the immune system. Immunosuppression is known to be produced by chemotherapy. Several cytostatic drugs including cyclosporin A, cyclophosphamide (CPS), azathioprine, and prednisone, used in transplantation, are considered immunosuppressive drugs (*18*). Cytostatic drugs were shown to be inadvertently responsible for various types of cancer and increased susceptibility to infections in organ transplant patients (*7,18*). Heavy metals are considered to be immunosupressive and are ranked according to their immunosuppressive properties as follows: mercury > copper > manganese > cobalt > cadmium > chromium (*19*). Many environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,7,8,-tetrachlorodibenzofuran (TCDF), polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), organochlorine insecticides, and alkylating agents were shown to have a potential for immunosuppression in experimental models (*15,18,20–26*). Overall, exposure to such environmental contaminants can result in immunosuppression leading to decreased resistance to infections in experimental animals and humans. This is particularly true for systemic exposure to heavy metals such as arsenic, cadmium, lead, mercury, nickel, and organotins, which adversely impair the immune response and decrease host resistance to infectious agents and cancers (*19*). However, host defense mechanisms against various infectious agents involve different components of the immune system. Therefore, interpretation of experimental data is complicated when only a limited number of infectivity assays are used. For example, CPS was shown to affect host resistance in several viral and bacterial infections, except for herpes virus-2 (HSV-2) and *C. neoformans* for which even increased resistance in CPS-exposed animals was reported (*27*). Therefore, data from several different experimental bacterial and viral infectivity models and experimental tumor models are often needed to conclusively demonstrate potential chemically induced alterations in host defense mechanisms.

It is generally accepted that efficient modification of the host's antitumor responses through augmentation or restoration of existing effector mechanisms might be beneficial (*28,29*). These beneficial agents are called biologic response modifiers (BRMs) and produce immunorestoration or immunopotentiation. BRMs have been successfully applied to individuals whose immune systems are compromised due to malnutrition, aging, acute and chronic infections, and cancer (*29*). BRMs, including interferons, interleukins, levamisole, and dithiocarbamate derivatives, have been shown to potentiate the immune response in immunopharmacologic studies (*28,29*). Other immunopotentiating/immunorestoring drugs such as isoprinosine, muramyl dipeptide, azimexon, bestatin, tuftsin, and pyrimidinoles have also been reported to alleviate symptoms or to shorten the duration of disease (*29*).

The beneficial effects derived from the therapeutic application of BRMs, however, should be clearly discriminated from the uncontrolled and undesired immunopotentiating effects produced by several environmental chemicals such as cadmium, lead, carbamate pesticides, skin sensitizers, and autoimmunity-inducing drugs (*19,30–32*). Such responses can be classified as hypersensitivity or autoimmunity. Chemical-induced hypersensitivity, manifested primarily as a contact sensitization, is defined as an undesired disproportionate increase in the adaptive immune response following repeated exposure to a chemical (*33*). There are essentially four major types of hypersensitivity responses, three of which are mediated by the anaphylactic immunoglobulins (Ig) of the IgE or IgG isotype, and the fourth, is a so-called delayed-type hypersensitivity (DTH), which is mediated by a subpopulation of T lymphocytes (*33*).

Substances capable of eliciting hypersensitivity responses in presensitized individuals are called allergens. Allergens elicit hypersensitivity responses by triggering the effector mast cell in a two-stage process in which the allergen-induced anaphylactic antibodies bind to cell-surface receptors and activate the mast cells to release mediators such as biogenic amines, lipid mediators, and cytokines. The IgE-mediated hypersensitivity, classified as type I hypersensitivity and commonly known as allergies, such as hay fever and certain types of asthma, can be causally linked to exposure to pharmacologic agents (*34*).

Several occupational allergies, including asthma, can be also induced by chemicals such as platinum halide salts, antibiotics, diisocyanates, epoxy resin activators, ammonium persulfate, and heavy metals such as beryllium (*34,35*). Thus, these substances must be considered immunotoxic.

There are situations in which individuals exhibit clinical characteristics of immediate-type hypersensitivity responses without a proven involvement of any anaphylactic antibodies of the IgE or IgG isotype (*36,37*). These symptoms may range from skin rashes to, occasionally, fatalanaphylaxis. This so-called pseudoallergy, although not antibody-mediated, is suspected to be mediated by sensitized lymphocytes and associated cytokines. Pseudoallergy may be induced by many chemicals including certain polypeptide hormones, antibiotics, intravenous anesthetics, and radiographic contrast media (*37*).

The combination of xenobiotics with host tissue constituents in certain susceptible individuals can lead to the production of antibodies directed against tissue or cell surface antigens. Tissue injury and disease can result from recruitment and activation of inflammatory cells and complement activation. This type of response is called type II hypersensitivity (*36,37*).

The conjugation of certain low molecular weight substances to host proteins can elicit high levels of circulating IgG or IgM antibodies, which can lead to the formation and deposition of immune complexes, particularly in blood vessels, and can cause tissue injury and disease through the recruitment and activation of inflammatory cells and complement activation. Immune complex-mediated diseases are classified as type III hypersensitivity (*36,37*).

Finally the type IV hypersensitivity reaction DTH is mediated by activated macrophages and sensitized T lymphocytes. A classical example of DTH is allergic contact dermatitis, which can be induced by a number of environmental and occupational agents. For example, heavy metals such as chromium, cobalt, mercury, and nickel are known to be strong skin sensitizers in experimental animals (*38*). Among the heavy metals, nickel and beryllium have been shown to elicit DTH responses in susceptible individuals (*35*).

Autoimmunity, sometimes called autoallergy is defined as the body's immune system turning against itself by producing lymphocyte subsets that are reactive with the host's self-components. These lymphocytes may be B-cells that upon activation may synthesize and secrete autoantibodies, T-cells that attack and destroy target organs, or a combination of both. Basically, a multifactorial etiology is involved in inducing the production of

altered-self structures of the host, which consequently might trigger autoimmune reactions (*31,32*). Factors such as age, nutritional status, and the genetic constitution may predispose the individual to autoimmunity (*31*). Moreover, environmental and occupational agents have the potential of inducing autoimmunity in susceptible individuals. Indeed, chemicals may combine with and modify the host's tissue antigens (*32*), trigger the release of tissue components that are not normally present systemically, or cause autoimmunity by directly affecting lymphocytes or macrophages. Humans exposed to gold salts and mercury-containing compounds have experienced immune complex glomerulonephritis, which may progress to interstitial immune complex nephritis (*39*).

Toxicologic End Points Predictive of Immunotoxicity

Review of routine toxicity data can provide an insight into the potential action of a particular chemical on the immune system (*40*); weight loss or reduced weight gain is a sign of toxicity that may complicate the interpretation of possible direct effects on the immune system. Alterations in adrenal weight and morphology may signal changes in stress-mediated adrenal glucocorticoid hormones and subsequent induction of lymphopenia or lymphoid depletion, especially in the thymus (*41*); alterations in the size and weight of the thymus reflected as a loss in cortical lymphocytes, often referred to as thymic atrophy, are important especially in subchronic studies conducted on young animals in which the thymus is at its maximal size (*42*). Reduction in the weight of the spleen, reflected microscopically in the reduction in size of germinal centers, often follows a reduction in the periarterial lymphoid sheath portion as well as reduction in the size of the gut-associated lymphoid tissue (Peyer's patches) (*43*). Bone marrow changes manifested in sustained anemia and an increase in the myeloid/erythroid ratios can be attributed to a selective effect on the erythroid stem cells and on erythroid maturation processes (*22,44*); changes in the cellular components of blood, especially in leukocyte counts, can be detected in subchronic studies. These may include an increase, a decrease, or shifts in cellular components during a differential count (*45*). Decreased levels of serum protein, due primarily to an increase in the albumin, lead to changes in the albumin/globulin ratio, changes in globulin levels normally due to a reduction in α-globulins, and total serum immunoglobulins (*45*).

Additional toxicologic indices, which can be useful in determining whether a chemical is potentially immunotoxic, include metabolic/biochemical interactions, such as the binding or sequestering of lipophilic xenobiotics to lipoprotein carriers (*46*), and subsequent distribution, activity, elimination, or bioactivation (*47*). For example, the immunomodulating chemicals, CPS and *N*-nitrosodimethylamine, require metabolic activation for their action on the immune system. Thus, immunosuppression can be demonstrated *in vitro* only in co-cultures of the immunocompetent cells with hepatocytes (*47,48*). Overall, a variety of structural changes in the immune system observed during subchronic and chronic toxicity studies can be useful indicators of a potential immunotoxic effect of chemicals and can be used as a base in designing further immunotoxicity studies.

Evaluation of Chemical-induced Immunotoxicity

The structural and functional complexity of the immune system requires that multiple parameters be examined for a comprehensive evaluation of chemically induced immunomodulation to be made.

Current guidelines for toxicity testing of chemicals published by the Organization of Economic Cooperation for Development (OECD) include bioassays for acute, short-term, and long-term toxicity, as well as tests for carcinogenicity, mutagenicity, teratogenicity, and reproductive and developmental toxicity (*49*). Regarding the immunotoxic potential of chemicals, the OECD guidelines in their present form are restricted to the quantitative assessment of total and differential leukocyte counts and histopathologic examination of the spleen. However, recent studies have shown that the present form of the OECD guidelines is not suitable for an adequate assessment of potentially immunotoxic chemicals (*50*). Currently, efforts are been made internationally to modify the OECD guidelines to include additional immune-related tests that may predict the immunotoxic potential of chemicals (H Koeter, personal communication). Meanwhile, a number of proposed guidelines and detailed methodology have been published for the assessment of chemically induced immunotoxicity using the mouse and the rat as the animal models (*4,6–8,51–54*). Due to the complexity of the immune system, a two-tier approach has been suggested. Tier I is usually a screen for potentially immunotoxic chemicals and contains assays that evaluate immunopathology, HMI, CMI, and NSI. Tier II consists of specific confirmatory immune tests and in-depth mechanistic studies. Tier II of the proposed guidelines for immunotoxicity testing also provides a comprehensive assessment of host resistance to challenge with syngeneic tumors and bacterial, viral, or parasitic agents (*6,7*). The inclusion of infectivity assays into Tier II is important since a higher incidence of mortality due to infection was observed in exprimental animals, wildlife, and fish whose immune systems were compromised following exposure to chemicals as compared to the incidence in nonexposed animals (*20,55–57*). Also, early epidemiologic studies indicated that decreased antiviral resistance in children could be a consequence of immunosuppression due to massive spraying of forestry insecticides or chemical carriers in the insecticide formulations (*21*). Generally then, a series of quantitative and functional immunologic assays are designed to test for adverse effects of chemicals on the immune system.

Progress in intra- and interlaboratory validation of immunotoxicologic methods regarding their potential for predicting immune effects of xenobiotics revealed that the plaque-forming cell (PFC) technique, combined with the assays for natural killer (NK) cell activity, the analysis of lymphocyte subpopulations, the mitogen-induced B- and T-cell proliferation, and the DTH response, had a high degree of concordance with the predicted immunotoxic properties of chemicals (*6*). These functional assays, when combined with other functional and quantitative techniques, can be powerful tools in localizing the type of cell function targeted by immunotoxic chemicals. For example, quantification of the humoral response at a single cell level by the PFC method combined with quantification of specific antibody production using the enzyme-linked immunosorbent assay (ELISA) technique (*50*), and investigations regarding the function of the antigen presenting cells following exposure to the organochlorine pesticide dieldrin revealed that the observed effect on antibody production was due to a chemical-related effect on antigen presentation by the mononuclear phagocytic cell (*23,24*). Overall, it was concluded that a good correlation existed between changes in the immune tests and altered host resistance.

No single immune test, however, could be identified that was fully predictive for altered host resistance, although most assays were relatively good indicators (*6,7*) for immunotoxic effects of chemicals.

While the majority of quantitative and functional immune assays were developed and validated for use in the mouse and rat (*4,6,49*), the increased use of other species in immunotoxicity studies has prompted the development and validation of a number of immune assays in species other than rodents. Several of these assays were shown to be good predictors of the immunotoxic potential of environmental chemicals and included assays for nonhuman primates (*15,25,26,58,59*), dogs (*60–64*), marine mammals (*65*), and fish (*2,57,65*). Several new end points of potential usefulness in the assessment of chemically induced immunotoxicity have been proposed in recent years (*4,6,16,17,42*). These include quantification of cytokines such as interleukins, interferons, and other factors secreted by lymphocyte and monocyte/macrophage cells. Other advanced histologic techniques, including immunohistochemistry, *in situ* hybridization, electron microscopy, confocal laser scanning microscopy, and computerized image analysis have been introduced for better understanding of drug-related immunotoxicity (*66*).

Methods for evaluating chemical skin sensitizers and chemically modified self-antigens that use the guinea pig as the animal model include the occluded patch test (Buchler test), nonadjuvant topical guinea pig skin sensitization immediate hypersensitivity reactions, and guinea pig maximization test (*67–69*). The local lymph node assay was primarily proposed as a predictive test for the identification of contact allergens (*30,70*). The mouse ear swelling test was developed as an alternative test for delayed contact hypersensitivity (*71*). The popliteal lymph node (PLN) enlargment in mice and rats was proposed as a predictive test of autoimmunity-inducing drugs (*31,32*). While the mechanism of PLN enlargment by low-moleculer autoimmunity-inducing drugs is not well understood, results obtained with the graft-versus-host technique and quantification of activated $CD4^+$ and $CD8^+$ T-cell subsets by flow cytometry provided unequivocal evidence for chemical-induced dysregulation leading to autoimmune phenomena (*14,31,72*).

Finally, development of *in vitro* models for immunotoxicity testing would be clearly advantageous, especially in studies involving human peripheral blood lymphocytes directly exposed to the chemical of interest (*17*). This approach, however, is restricted by the limited number of available *in vitro* techniques and the frequently observed lack of correlation between *in vivo* and *in vitro* results and is limited only to highly immunosuppressive chemicals that do not require metabolic activation (*17,47,48*). A good example of new approaches in the *in vitro* studies is the increasing appreciation of the relevance of programmed cell death (apoptosis) (*73*). The apoptotic cell death assay was recently applied to lymphoid cell lines to study the effects of chemical immunotoxicants on programmed cell death (*74,75*).

Summary

A general overview of immunotoxicology has been presented. The immune system is a structurally and functionally complex system composed of several cell populations and organs strategically placed throughout the host's body. Maturation of the immune system depends largely on its encounter with exogenous agents including microbial infections while regulation of the immune system is a function of cell–cell interactions mediated by a variety of adhesion molecules and endogenously produced substances including the cytokines, which interact with receptors found on the cell surface. The structural and functional integrity of the immune system are crucial in performing its protective role against pathogenic agents and the development of cancer. Thus, any chemically induced perturbation of the host's immune system can compromise its protective capacity and may lead to adverse health consequences for its host. An array of immunological methods have been developed and validated during the last decade. These methods have been used extensively to study quantitative and functional aspects of the immune system in experimental animals and, to a lesser extent, in humans accidentally exposed to potentially immunotoxic chemicals either occupationally or by eating contaminated food. These studies indicated that chemicals of environmental concern can affect the immune system adversely and have assisted in unraveling the mechanisms of action for some of these chemicals. Such studies have contributed to the process of evaluating the potential risk chemicals may pose to human health. Although an impressive progress has been made in the field of immunotoxicology during the last decade, much work remains to be done in *in vitro* immunotoxicology and in the application of methods developed in experimental animals to the human situation. Such efforts combined with the many established immunotoxicology principles and methods will, undoubtedly, increase the degree of confidence in data extrapolation from experimental animals to humans.

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